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Laboratory approach to study toxico-pathological interactions in the honey bee *Apis mellifera*

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1. Introduction

Pesticides and pathogens are two categories of environmental stressors that may contribute to the decline of honey bee populations (vanEngelsdorp and Meixner, 2010). However, if their separate impacts on the honey bee are relatively well studied, knowledge on their interactions are somewhat lacking. Pioneer studies on toxico-pathological interactions have been conducted on the association of Nosema and chronic bee paralysis virus (CBPV) with organophosphate, organochlorine and pyrethroid insecticides (Ladas, 1972; Bendahou et al., 1997). These studies focused on the acute exposure to insecticides regardless of their chronic toxicity. However, the introduction of systemic insecticides, such as phenylpyrazoles and neonicotinoids in the mid 1990's renders more relevant the studies on chronic exposures to pesticides by oral route. Since, Suchail et al. (2001) have reported a discrepancy between acute and chronic toxicity of the neonicotinoids imidacloprid and its metabolites showing a high toxicity at very low doses.

A new laboratory approach to study the chronic toxicity of insecticide has offered the possibility to explore the interactions between pathogens and pesticides during chronic exposures (Suchail et al., 2001). Studies on the joint exposure to Nosema and systemic insecticides have revealed that toxico-pathological interactions may elicit damaging effects on the bees, even when both stressors have no or limited effects on bee mortality (Alaux et al., 2010; Vidau et al. 2011). Two approaches have been used to study the effects of pesticide-pathogen associations. The first carries out simultaneous exposures to the pathogen and the pesticide and is particularly suitable to reveal antagonistic, additive and synergistic effects (Alaux et al., 2010). The second involves sequential exposures to the pathogen and the pesticide and is particularly relevant to investigate the sensitization to one stressor by another (Vidau et al., 2011; Aufauvre et al., 2012).

2. Materials

Honey bees

Traditionally, the effects of pesticides are investigated in honey bee foragers that are the individuals first exposed to pesticides. Considering the contamination of pollen and honey by systemic insecticides, all individuals may be potentially exposed by ingestion of a contaminated food. Thus, the exploration of the toxico-pathological interactions has also been studied in cohorts of young isolated bees of known age, which represent a relatively homogeneous biological material. A sufficient amount of honey bee colonies not infected by Nosema, as confirmed by PCR and using primers previously described (Martin-Hernandez et al., 2007), must be selected in order to obtain the desired number of emerging bees. To make the collect of emerging bees easier, queens can be isolated, 20 days before the starting of the experiment, using a queen excluder grid during 24 hours.

To fully sustain their physiological maturation after emergence, bees ingest pollen during the first days of their life. Pollen is the natural source of proteins for bees but the risk of contamination by pesticides cannot be ruled out (Chauzat et al., 2006; Mullin et al., 2010). A chemical analysis should normally yield information on the pesticides residues present in the pollen. However, the limit of detection of pesticides achieved with multi-residue methods are above 2 µg/kg for a large number of substances. Thus, a substance may be not detected but might still induce toxicity below its limit of detection. In addition, pathogens, notably Nosema and viruses, can be found in the pollen (Higes et al., 2008; Singh et al., 2010. For this

reason, pollen is replaced by yeast extracts for protein supply. Commercial protein supply can be used.

The day before starting the study, frames of sealed brood are sampled from colonies, put in boxes and placed in an incubator in the dark at 34°C with 80% relative humidity.

The day of the study, emerging honey bees (0-1 day) present in the boxes are collected, confined to laboratory cages (e.g. Pain type, 10.5x7.5x11.5 cm) in groups of 30-50, and maintained in the incubator for different periods of time at 30-32°C and 70-80% relative humidity. To mimic the hive environment, a little piece of wax and a Beeboost® (Pherotech, Delta, BC, Canada) releasing one queen-equivalent of queen mandibular pheromone per day, are placed in each cage.

Pesticide

Stock solutions of pesticides in 100% DMSO will be diluted to obtain the required concentration of pesticide and 0.1% DMSO final concentration in 50% (w/v) sucrose syrup.

Food supply:

Sucrose solution for experimental treatments (pathogens and pesticides) is made with sucrose and distillated water (50%; w/v). Proteins (Provita'bee) and candy (Apifonda®) can be purchased from beekeeping suppliers.

For more details on laboratory rearing methods see the chapter: Standard methods for maintaining adult *Apis mellifera* outside in cages under *in vitro* laboratory conditions.

3. Joint action of pathogens and pesticides

1. The day of the study, emerging honey bees (0-1 day) present in the boxes are collected and distributed in different experimental groups: (i) uninfected controls, (ii) infected with the pathogen only (*e.g. N. ceranae*), (iii) uninfected and chronically exposed to the pesticide at different doses, and (iv) infected with the pathogen and chronically exposed to the pesticide at different doses. Emerging bees can be handled relatively easily because they are quiet and neither sting or fly.

2. Honey bees are first individually infected by feeding with 3 µl of a freshly prepared 50% (w/v) sucrose solution containing the appropriate *inoculum* of the pathogen. Feeding is performed by holding each bee with its mouthparts touching the sucrose droplet at the tip of a micropipette (Malone and Gatehouse, 1998). This induces the extension of the proboscis and allows the bees consuming the entire droplet. Non-infected bees are similarly treated with the sucrose solution devoid of pathogen.

3. Bee are then confined to laboratory cages in groups of 30-50, and maintained in the incubator at 30-32°C and 80 % relative humidity.

4. Honey bees are chronically exposed to pesticides for different periods of time by ingesting *ad libitum*, 10 h per day, 50% sucrose syrup containing, 1% (w/v) proteins, the pesticide at the appropriate concentration and 0.1% DMSO. The remaining 14 h, bees are fed with Candy and water *ad libitum*.

During the experiment, each cage is checked every morning and dead honey bees are removed and counted. The food, containing or not the pesticide, is freshly prepared and renewed daily. The actual insecticide consumption is quantified by measuring the daily amount of sucrose syrup consumed per bee.

4. Sensitization to pesticides by a previous exposure to pathogens

1. Bees are distributed in different experimental groups: (i) uninfected controls, (ii) infected with the pathogen only (*e.g. N. ceranae*), (iii) uninfected and chronically exposed to the pesticide at different doses 10 days post-infection (d.p.i.), and (iv) infected with the pathogen and chronically exposed to the pesticide at different doses 10 d.p.i.

2. Honey bees are first individually infected with the pathogen (see above). If studies are conducted on emerging bees, go to step 3. If studies are performed on aged bees, go to step 5.

3. *Studies on emerging bees.* Honey bees are individually infected by feeding with 3 µl of a freshly prepared 50% (w/v) sucrose solution containing the appropriate *inoculum* of pathogen. Emerging honey bees are then fed during 10 days with 50% (w/v) sucrose syrup supplemented with 1% (w/v) proteins 10 h per day and thereafter with candy and water *ad libitum* 14 h per day. Each day, feeders are replaced and the daily sucrose consumption is quantified.

4. Ten days after infection, honey bees are then chronically exposed for 10 days to the pesticide by ingesting *ad libitum*, 10 h per day, 50% (w/v) sucrose syrup containing 1% proteins, the pesticide at the appropriate concentration and 0.1% DMSO. Honey bees not exposed to insecticides are fed *ad libitum* with sucrose syrup containing 1% proteins and 0.1% DMSO. Then, bees are fed with candy and water *ad libitum* 14 h per day.

5. *Studies on aged bees*. At a given post-emergence time, caged bees are CO_2-anaesthetized, put individually in infection boxes consisting of ventilated compartments (3.5x4x2 cm) and starved for 2 h. Each compartment is supplied with a tip containing the appropriate inoculum of pathogen in 3 µL of sucrose syrup (non-infected bees are similarly treated with sucrose syrup devoid of pathogen).

6. Infection boxes are placed in the incubator and 1 h later, bees that have consumed the total pathogen solution are again encaged (50 bees per cage). Bees are then fed during 10 days with 50% (w/v) sucrose syrup supplemented with 1% (w/v) proteins 10 h per day and thereafter with candy and water *ad libitum* 14 h per day. Each day, feeders are replaced and the daily sucrose consumption is quantified.

7. Ten days after infection, honey bees are then exposed for 10 days to the pesticide (see point 4 above).

Throughout both types of experiments, each cage is checked every morning and dead honey bees removed and counted. The food, containing or not the pesticide, is freshly prepared and renewed daily. The actual insecticide consumption is quantified by measuring the daily amount of sucrose syrup consumed per bee.

At the end of the experiment (20 d.p.i.), surviving honey bees can be subjected to investigations or may be quickly frozen and set aside for subsequent analysis.

Notes

To analyze honey bees at a second post-infection time, the number of cages for each modality must be multiplied by two.

To avoid any bias due to the weather or season on bee physiology, mortality, physiological and chemical investigations should be performed at the same time.

Honey bees must be handled with a soft insect holding forceps to avoid physiological damages.

The experimental design may be modified to change the day of infection, the starting day and the duration of exposure to pesticide, and the sequence of exposure to stressors.

It is proposed to expose the bees to the pesticide 10 h per day in order to avoid overexposure not compatible with environmental exposures (Suchail et al., 2001). However, bees can be exposed continuously to the pesticide.

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